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Research Article

Isolation and Characterization of Plant-Derived Exosome-Like Nanoparticles (PDEN) from Sapodilla Fruit (*Manilkara zapota*) as an Antioxidant Agent in 1BR3 Cell Line

Anastasia Noeng[1], Anggraini Barlian[1,2*]

[1]*School of Life Science and Technology, Bandung Institute of Technology, Bandung 40132, Indonesia*
[2]*Scientific Imaging Center, Bandung Institute of Technology, Bandung 40132, Indonesia*

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ABSTRACT

Plant-derived exosome-like nanoparticles (PDENs) have emerged as promising natural nanocarriers with potential applications in biomedicine and cosmetics due to their biocompatibility, stability, and intrinsic bioactivity. The objective of this research is to isolate, characterize, and analyze the effects of MZ-PDENs on 1BR3 cells. MZ-PDENs were isolated using filtration, differential centrifugation, and polymer-based precipitation. MZ-PDENs were characterized for their physiochemical properties, size, shape, and antioxidant activity. *In vitro*, bioactivities were conducted by measured cytotoxicity, proliferation assays, UVB protection, and migration assays. Results showed that MZ-PDENs possess strong antioxidant properties, are efficiently internalized by cells, and promote skin cell regeneration after UVB-induced damage. These findings suggest that MZ-PDENs have the potential as antioxidant agents for cosmeceutical applications and warrant further investigation *in vivo* models.

1. Introduction

The average Indonesian is exposed to free radicals, such as UVB rays, for approximately one year. The UVB can result in alterations to the skin's appearance, such as hyperpigmentation, wrinkles, fine lines, and an increased risk of developing skin cancer. A study by Gao *et al.* (2022) demonstrated that UVB exposure can damage the deeper layers of the dermis. In response to the developments in the cosmeceutical industry, researchers have been seeking to identify naturally occurring chemical compounds that can be employed in the treatment of skin problems. One of the compounds that remains to be identified is exosomes derived from animal cell culture, which has been used for over a decade to regulate cells (Mistry *et al.* 2012). However, they are now being considered as an alternative source of nanoparticles. PDEN functions as a carrier agent or nanocarrier, such as an antioxidant compound, offering a potential solution for addressing dermatological issues resulting from UVB exposure (Choi *et al.* 2024).

The sapodilla fruit, in both its fresh and dry forms, is known to be a rich source of antioxidants. A variety of studies have been conducted on the sapodilla, utilizing samples from the leaf, fruit, and even the branch. The sapodilla has been utilized as a source of antioxidants for various purposes, including antimicrobial, anticancer, anti-inflammatory, anti-diabetic, and anti-arthritic applications, as well as preventing hyperpigmentation (Rahal *et al.* 2014; Chunhakant and Chaicharoenpong 2019). Cortes *et al.* (2022) demonstrate that antioxidants, such as vitamin C, possess the capacity to inhibit free radicals. Vitamin C, also known as ascorbic acid, has

* Corresponding Author
E-mail Address: aang@itb.ac.id

been shown to inhibit melanogenesis and stimulate the synthesis of collagen type I (Cortes *et al.* 2022). However, the use of vitamin C in cosmetic products has been associated with the development of acute dermatitis in some cases and may be harsh on sensitive skin (Assier *et al.* 2014).

2. Materials and Methods

2.1. Uptake of MZ-PDENs by 1BR3 Cells

Sapodilla was collected from the Cisitu farm in Sumedang, Indonesia—approximately 500 mL of fresh juice was obtained from 770 grams. We followed two procedures from Emmanuela *et al.* (2024) and Kalarikkal *et al.* (2020) with slight modifications. Finally, the pellet was diluted in aquabidest, followed by filtration using a polyether sulfone syringe filter with a 0.22 µm pore size. Dynamic Light Scattering (DLS) was performed using a Nano Particle Analyzer (Horiba SZ-100) to evaluate the size distribution, zeta potential value, storage temperature, and duration of MZ-PDENs. For assessment of storage and duration, fresh MZ-PDENs were stored at 4°C and -20°C for four weeks. The total protein concentration of MZ-PDEN was measured using PierceTM BCA Protein Assay kits (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocol. The morphology of MZ-PDENs was characterized by a low transmission electron microscope (HT7700, 120 kV) using the negative staining method. Analysis using gas chromatography followed the method described by Stanly *et al.* (2020). This assessment was conducted at Laboratorium Central Universitas Padjadjaran.

2.1.1. DPPH Assay for Antioxidant Activity of MZ-PDENs

The free radical scavenging activity of MZ-PDENs is measured using a protocol by Shimamura *et al.* (2014). Amount 2 mg MZ-PDENs and ascorbic acid diluted separately by methanol 1,000 µL. Mixed 90 µL DPPH reagent (0.2 mM) to 10 µL MZ-PDENs or ascorbic acid in an ELISA plate and incubated at room temperature for 30 minutes. Ascorbic acid was used as the standard for this assay. The sample and standard were measured at 517 nm using a spectrophotometer (Biochrom EZ Read 2000) and Galapagos software. DPPH reagent with methanol was used as a control, and methanol alone was used as a blank. The DPPH antioxidant activity was calculated using this formula:

$$\text{Radical scavenging}(\%) = \frac{\text{Absorbance control} - \text{Absorbance sampel}}{\text{Absorbance control}} \times 100\%$$

2.1.2. Uptake of MZ-PDENs by 1BR3 Cells

MZ-PDENs were stained with the PKH67 Fluorescent Cell Linker Kit (Merck, Germany), as described in a study by Kusnandar *et al.* (2024). 1BR3 cell lines were grown on a confocal dish for 1 day. Cells were washed three times with PBS to remove the media. Semi-confluent 1BR3 cells incubated with media and PDEN labeled PKH67 at 37°C under 5% CO_2. After 60 minutes of incubation, uptake was stopped by washing with PBS, followed by the addition of PFA 4%. Cells were stained with DAPI (Sigma-Aldrich, USA) for nuclear staining and visualized using a Confocal Laser Scanning Microscope (CLSM) from Olympus, specifically the FV-1200 and FV-3000 models.

2.2. Cytotoxicity and Cell Proliferation Assay

Cell line 1BR3 was used as a model of human dermal fibroblasts in this experiment. It was verified by the European Collection of Authenticated Cell Culture (ECACC), UK Health Security Agency, Porton Down, Salisbury, UK. This assessment followed the method by Natania *et al.* (2024) with slight modifications. 1BR3 cells incubated for 72 hours with MZ-PDENs at concentrations 0, 2.5, 5, 7.5, 10, 15, 50, and 100 µg/mL and followed by proliferation assay 1BR3 cells incubated for 1, 3, 5, and 7 days with MZ-PDENs at concentration 0, 2.5, 5, 7.5 µg/mL. The absorbance was measured at 595 nm using a microplate reader and Bio-Rad software.

2.3. Cell Migration Assay

1BR3 cells from Sigma Aldrich, approximately 100,000/well, were seeded in triplicate in 24-well plates. Cells were cultured for 1 day until confluent and then pretreated with a mix of DMEM and MZ-PDENs at concentrations of 0, 2.5, 5, and 7.5 µg/mL for 24 hours. Scratches were made in the cell monolayer using 10 µL pipette tips. The observation was captured at 0, 6, 12, 24, 30, 36, and 48 hours using an inverted microscope with a Nikon camera and NIS Elements software. The gap between cells was later measured using ImageJ software (Rodriguez-Menocal *et al.* 2012; Kusnandar *et al.* 2024).

2.4. Cell Viability Assay After UVB Exposure

1BR3 cells, approximately 5,000/well, were seeded triplicate in 96 well plates and incubated for 24 hours as a pre-treatment with MZ-PDEN at concentrations 0, 2.5, 5, and 7.5 µg/mL. Cells were treated with UVB radiation at 100 mJ/cm² and incubated for 1, 3, and 5 days with the same concentration of MZ-PDENs. The number of viable cells was quantified by MTT reagent (5 mg/mL in PBS) conversion assay (Sigma-Aldrich, USA). Cells were incubated with MTT reagent mix in DMEM LG (1:9) for 4 hours at 37°C under 5% CO_2. Thereafter, cells were fixed and solubilized with 100 µL of DMSO. The absorbance was measured at 595 nm using a microplate reader and Bio-Rad software.

3. Results

3.1. Isolation and Characterization of MZ-PDENs

The isolation of sapodilla fruits was conducted after they had ripened, as processing unripe fruits with a juicer is challenging due to their considerable latex content. The filtrate was subjected to analysis using dynamic light scattering (DLS), which demonstrated that the average diameter of the isolated MZ-PDENs was 191.2±0.174 nm, with precipitation by PEG6000 at a concentration of 5%. The results indicate that at a concentration of 5% (w/v), the size of the MZ-PDENs did not exceed 300 nm, and the MZ-PDENs exhibited a relatively homogeneous size distribution. The zeta potential of the MZ-PDENs was determined to be -25.3 mV through DLS analysis. TEM imaging showed that MZ-PDENs possess a spherical shape (Figure 1B).

The optimization of MZ-PDENs storage conditions was conducted by measuring their stability at 4°C and -20°C over a four-week period in aquabidest as the solvent. As shown in Figure 1C, MZ-PDENs stored at 4°C for four weeks exhibited no significant change in diameter compared to those stored at -20°C. It is recommended that MZ-PDENs be stored at 4°C to stabilize them for up to four weeks. The GC-MS analysis, as shown in Table 1, identified 23 peaks in MZ-PDENs, with the two most prominent peaks corresponding to compounds olean-12-en-3-ol acetate (3 beta) and β-amyrin.

3.1.1. DPPH Assay for Antioxidant Activity of MZ-PDENs

The results demonstrated that at a concentration of 500 µg/mL, ascorbic acid exhibited a higher inhibitory effect on free radicals than MZ-PDENs, with an 80.05% radical scavenging ability compared to MZ-PDENs 12.58% inhibition (Figure 2). The IC_{50} of ascorbic acid was 215.528 µg/mL, while the IC_{50} of MZ-PDEN was 1688.255 µg/mL. Indicating that the IC_{50} of MZ-PDENs is eight times lower than that of ascorbic acid.

3.1.2. Uptake of MZ-PDENs by 1BR3 Cells

As shown in Figure 3, MZ-PDENs labeled with PKH67 were successfully observed to enter cells after a 60-minute incubation period. As illustrated in Figure 3B and C. MZ-PDENs are observed to be present within the cells, particularly in the cytoplasmic region.

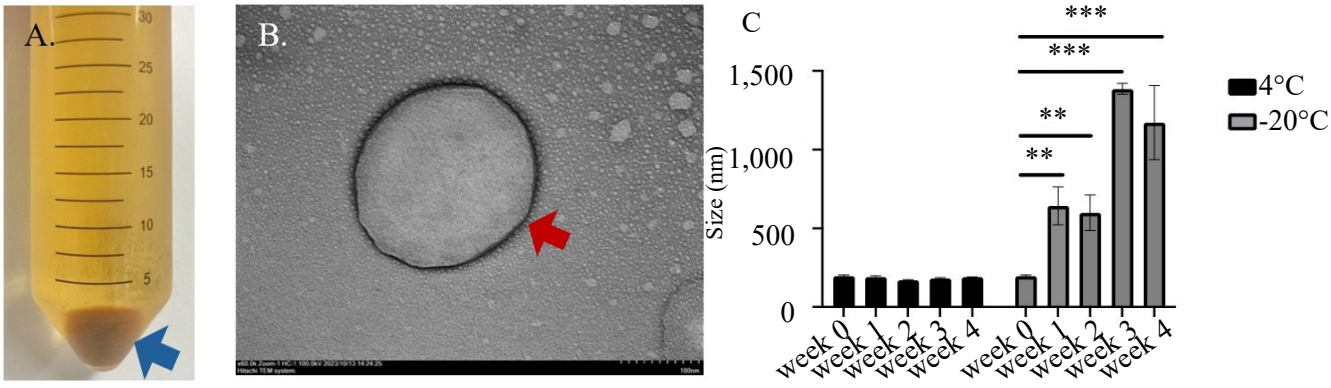

Figure 1. Characterization of PDENs isolated from *Manilkara zapota*. (A) MZ-PDENs in the form of brown pellets (blue arrows) after PEG6000 precipitation, followed by centrifugation, (B) MZ-PDENs morphology by TEM imaging (red arrows refers to MZ-PDENs), (C) effect of storage duration and temperature on the stability of MZ-PDENs. Data presented as mean ± standard deviation, (*significancy <0.05, significancy **<0.01, significancy ***<0.001)

Table 1. Profiling compounds for MZ-PDENs by GC-MS analysis

Area	RT	Area sum (%)	Compounds
319.34	11,066	0.06	3-Tosyl sedoheptulose
545.77	13,036	0.1	4-Hydroxymethyl-3,3,4-trimethyl-1,2-dioxetane, TMS derivative
794.44	8,663	0.15	3,7-Diacetamido-7H-s-triazolo[5,1-c]-s-triazole
992.55	24,918	0.19	2,3-Anhydro-d-galactosan
1197.84	39,749	0.22	1,2-Bis (trimethylsilyl) benzene
1328.29	28,226	0.25	Hexanedioic acid, mono(2-ethylhexyl) ester
1585.29	8,018	0.3	2,3-Anhydro-d-galactosan
1586.31	41,335	0.3	Arsenous acid, tris(trimethylsilyl) ester
2498.34	44,482	0.47	Tris(tert-butyldimethylsilyloxy)arsane
3187.53	30,469	0.6	Phthalic acid, dodecyl 2-methoxyethyl ester
3272.67	28,325	0.61	2-Methyl-3-decanol
3458.49	3,831	0.65	2,6,6-Trimethyl-bicyclo[3.1.1]hept-3-ylamine
3527.85	9,53	0.66	Alanyl-beta-alanine, TMS derivative
3603.19	4,809	0.67	Undecanal
4098.27	31,485	0.77	Oxirane, [(hexadecyloxy)methyl]-
4521.51	20,681	0.85	n-Hexadecanoic acid
4697	3,409	0.88	Imidazole, 2-amino-5-[(2-carboxy)vinyl]-
5154.9	19,913	0.96	Pentadecanoic acid, 14-methyl-, methyl ester
5451	34,409	1.02	Di-n-decylsulfone
5882.01	10,385	1.1	d-Glycero-d-tallo-heptose
32425.38	44,122	6.07	1,4-Bis(trimethylsilyl)benzene *)
136602.92	42,574	25.55	beta-Amyrin *)
307814.33	43,144	57.58	Olean-12-en-3-ol, acetate, (3beta) *)

*Compound with an abundance above 2%

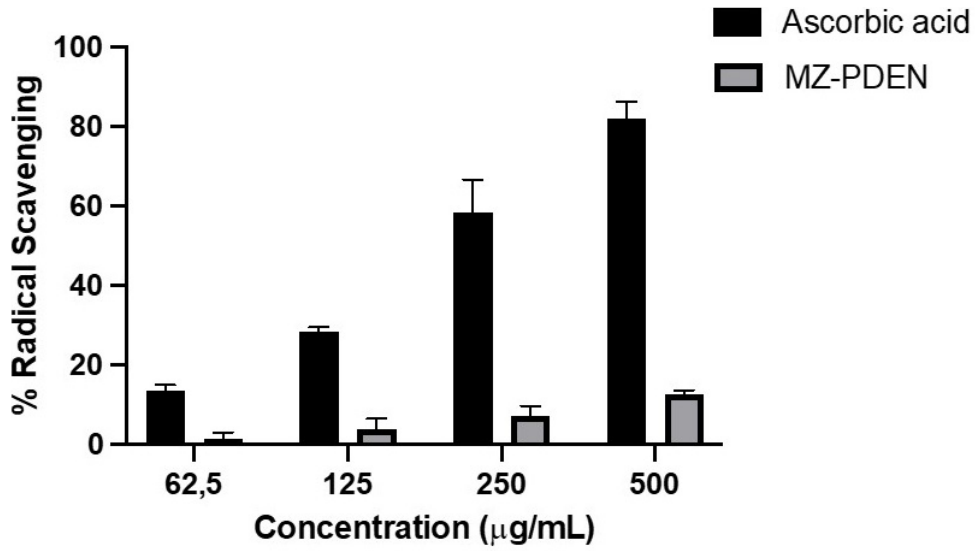

Figure 2. Radical scavenging activity of ascorbic acid and MZ PDENs used DPPH assay method. Both ascorbic acid and MZ-PDENs using differential dilutions to measured inhibition activity of free radicals

3.2. Cytotoxicity and Cell Proliferation

As shown in Figure 4, MZ-PDENs were non-toxic to the 1BR3 cells across the tested concentration range, with the lowest cell viability remaining above 80%. The concentrations for further *in vitro* assays were selected based on the results of the cytotoxicity tests. The concentrations of 2.5, 5, and 7.5 µg/mL proceeded to the next stage of testing. On the seventh day of measurement, the viability of the 1BR3 cells treated with MZ-PDENs at concentrations of 2.5 µg/mL was found to be significantly different from that of the control (Figure 5). These findings indicate that the incorporation of 2.5 µg/mL of MZ-PDENs enhances the proliferation of 1BR3 cells compared to cells that received no MZ-PDENs treatment.

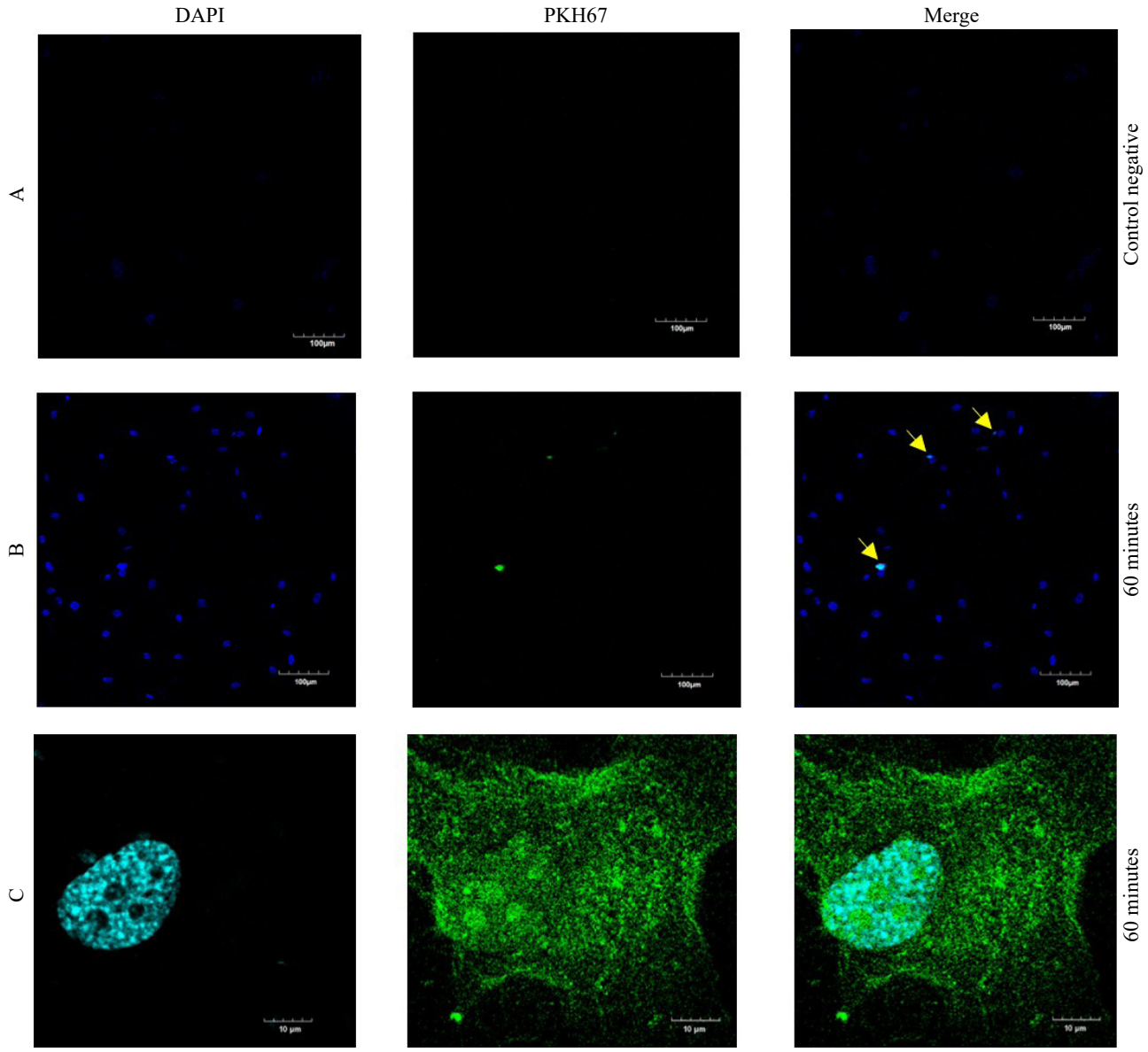

Figure 3. MZ-PDENs uptake by 1BR3 cells. (A) 1BR3 cells without MZ-PDENs, (B) MZ-PDENs labelled with PKH-67 (green, indicated by yellow arrows); nuclei labelled with DAPI (deep blue), uptake MZ-PDENs visualized by using CLSM Olympus FV-1200, (C) MZ-PDENs labelled with PKH-67 (green) and nuclei labelled with DAPI (cyan) after incubated 60 minutes, uptake MZ-PDENs visualized by using CLSM Olympus FV-3000

3.3. Cell Migration

As shown in Figure 6, the 1BR3 cells treated with 2.5 µg/mL of MZ-PDENs exhibited enhanced cell migration at 48 hours post-scratch in comparison to untreated cells. The closure percentage of the cells treated with 2.5 µg/mL MZ-PDENs was 77%, compared to a closure percentage of 57% in untreated cells (control).

3.4. Cell Viability After UVB Exposure

Following exposure to 100 mJ/cm^2, cells with added MZ-PDENs showed enhanced cell viability in comparison to the control without MZ-PDENs. By the fifth day, no significant difference in cell viability was observed between the 1BR3 cells treated with ascorbic acid and those treated with MZ-PDENs at all concentrations (Figure 7). This result indicates that

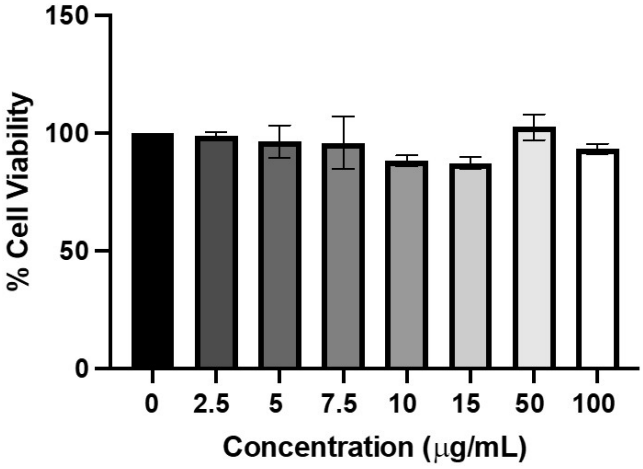

Figure 4. Effect of MZ-PDENs on cytotoxicity in 1BR3 cells measured by MTT assay. There is no differentiation among control and treatment by MZ-PDENs. Data presented as mean ± standard deviation, (p-value <0.05)

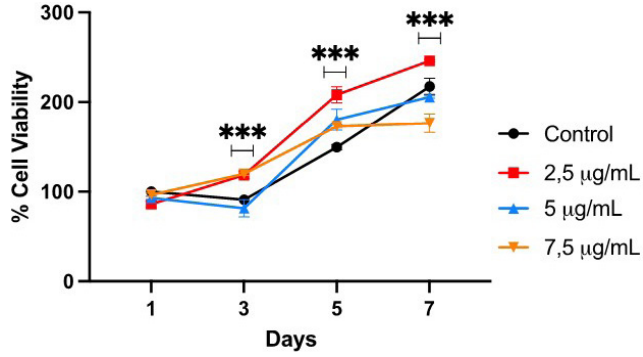

Figure 5. Effect of MZ-PDENs on proliferation in 1BR3 cells measured by MTT assay. Data presented as mean ± standard deviation, (p-value <0.001)

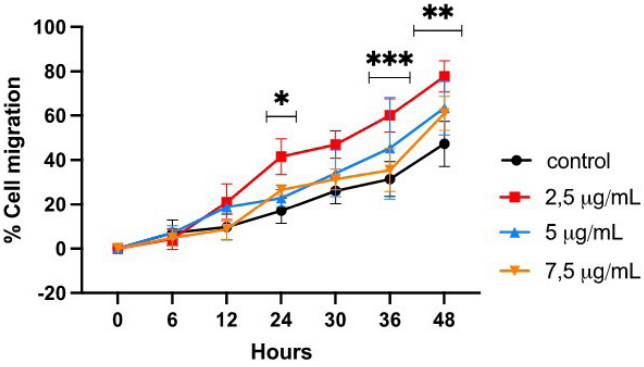

Figure 6. Total area migration of 1BR3 cells using method scratch assay for 48 hours. Data presented as mean ± standard deviation, (p-value <0.05)

MZ-PDENs at 2.5 µg/mL can protect cell viability with the same efficacy as ascorbic acid and more effectively than the untreated control (Figure 7).

4. Discussion

The optimal concentration of PEG6000, which affects the diameter of MZ-PDENs, is shown in Table 2. The size of the MZ-PDENs was selected following the report by Yi *et al.* (2023), which identified the typical range for PDENs as 100-500 nm. The previous study of black nightshade berries-derived exosomes used the same concentration of PEG6000 as that used for the isolation of MZ-PDENs (Emmanuela *et al.* 2024). However, the source of PDENs can affect the optimum concentration of PEG6000.

The zeta potential of PDENs itself ranges from -70 to 0 mV (Kim *et al.* 2022). DLS analysis revealed that the zeta potential of MZ-PDENs was comparable to that of the yam bean-derived exosome, which also exhibited a similar zeta potential (Kusnandar *et al.* 2024). The spherical morphology of MZ-PDENs (Figure 1B) is similar to that observed for PDENs from other sources, such as black nightshade berries, golden berries, and

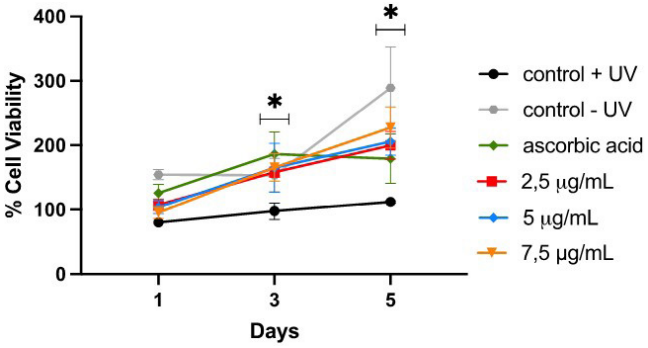

Figure 7. Cell viability of 1BR3 cells using MTT assay after exposure by UVB 100 mJ/cm^2. Data presented as mean ± standard deviation, (p-value <0.05)

Table 2. Size of MZ-PDENs after precipitation using a different concentration of PEG6000

PEG6000 concentration	5% (w/v)	10% (w/v)	15% (w/v)
Range MZ-PDENs size (nm)	171-246	151-655	105-655

yam beans (Emmanuela *et al.* 2024; Kusnandar *et al.* 2024; Natania *et al.* 2024). The protein concentration of MZ-PDENs was measured at 320 µg/mL. It is important to note that the measured protein concentration does not directly represent the number of particles (Dash *et al.* 2022).

Upon reaching their target cells, exosomes can enter the cell via three principal pathways: fusion with the plasma membrane, which allows the release of their cargo; endocytosis; or direct interaction with surface receptors (Krylova and Feng 2023)—from the results revealed that MZ-PDENs and yam bean-derived exosomes require approximately an hour to enter target cells (Kusnandar *et al.* 2024). Nevertheless, the uptake of PDENs by target cells varies depending on the source of PDENs and the target cell itself.

A study of exosomes revealed that they can be stored at -80°C for up to one year. It is recommended that exosome storage not occur at room temperature, as this will result in changes to the size and quality of the exosomes (Zhu and He 2023). The use of deionized water, such as aquabidest, as a solvent may influence the particle size, as deionized water may form ice crystals when stored below 0°C (Liu *et al.* 2022; Kusnandar *et al.* 2024). It is reasonable to conclude that storage of MZ-PDENs at -20°C may result in damage. PDENs derived from other sources, including black nightshade berries, golden berries, and yam beans, are stored under similar conditions to MZ-PDENs (Emmanuela *et al.* 2024; Kusnandar *et al.* 2024; Natania *et al.* 2024).

The antioxidant components with an abundance above 2% in MZ-PDENs were identified as olean-12-en-3-ol, acetate (3 beta), and β-amyrin (Table 1). Both compounds are triterpenoid derivatives (NCBI 2024a, 2024b). Triterpenoids are known to have antioxidant activity, as well as anti-aging and anti-melanogenic properties. They inhibit the action of tyrosinase, which is responsible for melanin production caused by hyperpigmentation (Desmiaty *et al.* 2021). The sapodilla fruit is known to contain a range of bioactive compounds, including ellagitannins, gallotannins, polyphenols, phenolic acids, vitamin C, flavonoids, and triterpenoids (Fernandez-Alba 2012; Chunhakant and Chaicharoenpong 2019; Bangar *et al.* 2022).

The formation of formazan crystal is dependent on the activity of dehydrogenase or oxidoreductase enzymes within the mitochondria in conjunction with the MTT reagents (Ghasemi *et al.* 2021). Cytotoxicity is a reliable indicator of cell viability, in accordance with the International Organization for Standardization (ISO) 10993-5:2009. According to this standard, a compound is considered non-toxic if cell viability remains above 80%. The results of MZ-PDENs were similar to those of papaya-derived exosomes, which were non-toxic to RAW 264.7 cells at concentrations up to 100 µg/mL (Iriawati *et al.* 2024). Subsequently, the concentrations of 2.5, 5, and 7.5 µg/mL MZ-PDENs were selected for further in vitro assessment.

It is well-established that exosomes can regulate gene expression in target cells and influence their biological activity (Liu *et al.* 2019). The results of the proliferation assay indicate that the addition of 2.5 µg/mL of MZ-PDENs facilitates enhanced cell proliferation in the 1BR3 cells in comparison to untreated cells after seven days (Figure 5). A comparable study conducted by Natania *et al.* (2024) using golden berries-derived exosomes showed that cell viability, as indicated by absorbance, was higher at a concentration of 7.5 µg/mL compared to HDF cells without golden berry-derived exosomes. Both MZ-PDENs and golden berry-derived exosomes have been proven to regulate the expression of target cells, thereby promoting cell proliferation to greater effectiveness than that observed in untreated cells.

The scratch assay is widely regarded as a reliable and cost-effective method for assessing cell migration. The cells will cover the wound area through a regulated proliferation and cell migration signaling mechanism (Liang *et al.* 2007), as shown in Figure 6. The results showed that 1BR3 cells treated with 2.5 µg/mL of MZ-PDENs exhibited enhanced cell migration at 48 hours post-scratch compared to cells not treated with MZ-PDENs. The concentration of MZ-PDENs has a similar result to that observed by Kusnandar *et al.* (2024), who used the same concentration of yam bean-derived exosomes and found that this enhanced cell migration more effectively than the untreated control after 24 hours. This statement provides evidence that MZ-PDENs and yam bean-derived exosomes are capable of regulating cell migration signaling for the wound healing process.

The use of ascorbic acid as a positive control in this study was based on its extensive utilization as an antioxidant agent within the cosmetic industry. A study conducted by Dikici (2023) and Wahyuningsih *et al.* (2020) found that ascorbic acid at a concentration of 50 µg/mL enhanced metabolic activity, cell growth, and collagen production in human dermal fibroblasts grown on polycaprolactone scaffolds and adipose-derived mesenchymal stem cells. Therefore, in this

study, a concentration of 50 µg/mL of ascorbic acid is used for this assay.

In a previous study by Choi *et al.* (2024), treatment with ginseng-derived exosomes at concentrations ranging from 1.25×10^8 to 2×10^9 particles/mL was found to enhance the viability of human epidermal keratinocytes (HaCaT) cells following UVB radiation exposure at 30 mJ/cm^2, compared to untreated HaCaT cells. Similar results showed that, despite MZ-PDENs exhibiting lower inhibitory effects and lower IC_{50} values than ascorbic acid, their efficacy on the viability of 1BR3 cells was comparable to that of ascorbic acid at 50 µg/mL and higher than the untreated control (Figure 7). These results indicate that MZ-PDENs may be a potential alternative to ascorbic acid in cosmetic formulations for individuals who are sensitive to ascorbic acid. The findings indicated that MZ-PDENs possess the potential to act as antioxidant agents in the development of cosmeceutical applications.

Conflict of Interest

The authors declare no conflict of interest.

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